Ethanol binge drinking during pregnancy and its effects on salivary glands of offspring rats: oxidative stress, morphometric changes and salivary function impairments

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ABSTRACT

Objectives: To investigate the biochemical and morphological effects of ethanol (EtOH) binge drinking during pregnancy on parotid glands (PG), submandibular glands (SMG), and saliva of offspring rats.

Methods: Pregnant Wistar rats (n = 8) were exposed to EtOH consumption (3 g/kg/day – 20 % w/v) for three consecutive days. The saliva of 40-day-old offspring rats was collected to determine amylase activity and total protein concentration. PG and SMG were collected to performe oxidative biochemistry, morphometric and immunohistochemistry analyses (Student's t-test, $p < .05$).

Results: EtOH consumption during pregnancy significantly decreased the total protein concentration and decreased amylase activity. In the PG, the EtOH group showed increased lipid peroxidation and decreased antioxidant capacity against peroxyl. In the SMG, the EtOH group showed increased lipid peroxidation and NOx metabolite levels. PG exposed to EtOH showed a decrease of acini, ducts, and total parenchymal area. SMG exposed to EtOH showed an increase in the total stromal area. The expression of CK-19 and Vimentin were found not different between groups.

Conclusions: For the first time, a three-day EtOH binge-drinking protocol during pregnancy is associated with oxidative stress and morphometric alterations in the salivary glands of offspring rats and with the functional reduction of the main salivary enzyme (amylase). Clinical relevance: EtOH consumption during pregnancy altered the morphology and physiology of the salivary glands of offspring rats.

1. Introduction

According to the World Health Organization (WHO), ethanol (EtOH) is the most widely consumed drug and causes six deaths per minute worldwide [1]. Considering the wide variation among WHO countries, the average EtOH consumption by individuals over 15 years old is 13.9 g/kg/day. A recurrent annual increase in EtOH consumption among adolescents and women (particularly in the reproductive age) has been demonstrated [1,2]. EtOH drinking patterns, which are determined by the number of drinks that an individual consumes, range from low (no more than three drinks on any single day and no more than seven drinks per week) to heavy (binge drinking on each of five or more days in the past 30 days) [3,4].

Five or more drinks for men and four or more drinks for women within two hours usually is considered binge drinking and leads to a blood alcohol concentration (BAC) of 0.08 g/dl or above [4]. Binge drinking is worldwide considered a health issue due to its association with unintentional injuries, violence, chronic diseases, sexually transmitted diseases, unintended pregnancy, miscarriage, stillbirth, fetal alcohol spectrum disorders, and other pregnancy-related diseases [5]. Since several risk factors are related to EtOH consumption, there is no safe dose during pregnancy [3].

Submandibular (SMG) and parotid glands (PG) are major salivary glands and play an essential role in the production of saliva components [6,7]. Salivary glands activity is responsible for the maintenance of oral homeostasis [8] Reduced or absent saliva flow (i.e., Sjogren¨ 's syndrome) may lead to inflammation of periodontal tissues, clinical attachment loss of periodontal tissue, and alveolar bone resorption, which indicates a case of periodontal disease [9].

Our research group has reported that EtOH binge drinking (3 g/kg) reduces the parenchyma and increases the stroma of salivary glands [10]. Decreased expression of proteins such as cytokeratin-19 (CK-19) and alpha-smooth muscle actin (α-SMA) was also found as a result of binge drinking [10,11]. It was recently demonstrated that the chronic consumption of a typical alcoholic Brazilian beverage named *cachaça* caused glandular atrophy, a decrease of alkaline phosphatase activity, and reduction of mucin synthesis in SMG of rats [12]. Rats submitted to EtOH chronic consumption were also found with microvasculature alterations on salivary glands capillaries and dystrophy of salivary

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ductal cells [13]. These effects were more pronounced within the first five days of alcohol consumption and no recovery was observed after 30 days [13, 14].

Considering that EtOH's effects on salivary glands morphology are evident, questions arise regarding the consumption pattern. Since it has been associated with unintended pregnancy [15,16], could binge drinking affect salivary glands? Thus, this study aimed to investigate the biochemical and morphological effects of a three-day EtOH binge-drinking protocol during pregnancy on SMG and PG of offspring rats.

2. Methods

Rattus Novergicus, 60 days old, were maintained in a room with standard conditions of temperature, 12 h reversed light/dark cycle (lights off 7:00 am), with free access to food and water. All procedures were conducted under compliance with policies of Federal University of Para Ethics Committee of Animals Use (CEUA N´º 6478190717) and following the guidelines of NIH *Guide for the Care and Use of Laboratory Animals* [17]. A single male was placed with one female from 08:00 am to 09:00 am, considering gestational day zero (GD0) determined by mating plug presence. Pregnant females were place in a single cage with nesting material after being weighted. Methodological summary is depicted on "Fig. 1".

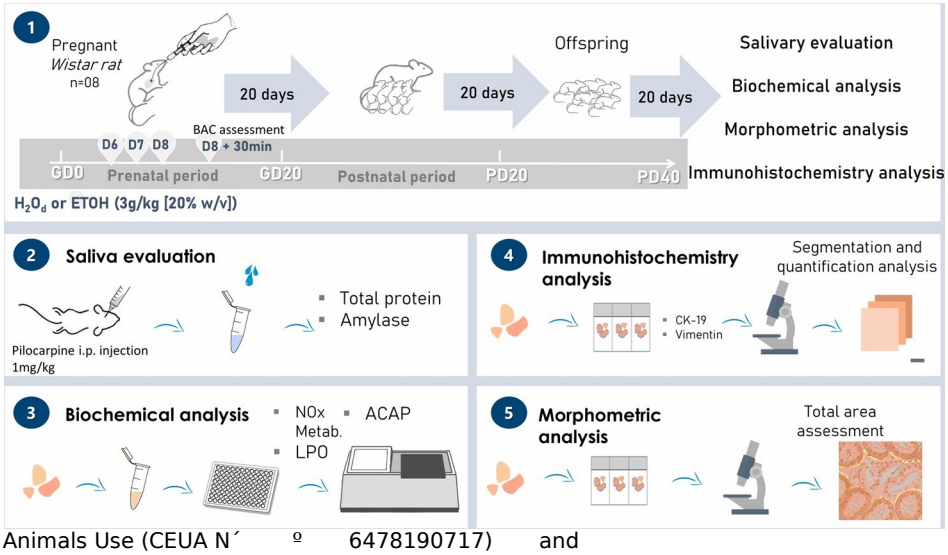
2.1. Treatment of groups and gestational period

Pregnant dams were randomly divided into two groups (simple randomization), control and EtOH group (n = 4, each). Both groups were treated in gestational days GD6, GD7, GD8, with distilled water (control) or EtOH 3.0 g/kg (20 % w/vol) through intragastric gavage. Number of animals and gestational days of exposure were selected based on Schambra et al. (2015) protocol [18] (SD 1.2, power of 80 %). These days represent the rat gastrulation and neurulation periods and involve salivary glands innervation morphogenesis, through neural crest cells migration [19]. After treatment periods, dams were kept under standard living conditions in single cages and have been weighted on GD13 and GD18. Two checkups after GD18 were performed daily for parturition verification. Day of birth was determined as postnatal day zero (PD0).

Offspring groups were assigned at PD7. At PD20 males and females' pups were weaned separated in different cages respecting treatment groups and weighted weekly until PD40.

2.2. Blood alcohol concentration analysis

Blood Alcohol Concentration (BAC) was obtained on GD8, after 60 min of alcohol intake, in the last three days of the binge drinking model exposure. Blood samples (Pregnant dams, 2 per group) were collected and transferred to heparinized tubes, and then stored at 4 °C until the EtOH concentration was measured. Briefly, homogenized blood sample (50 µL) was added to butanol as internal standard (50 µL) and analyzed by the head space gas chromatography (GC/MS) with Flame Ionization Detector (FID) on GC–MS Varian equipment (Walnut Creek, CA, USA), model CP3800, equipped with automatic injection of samples (CombPalm Series number 1210469). The capillary column (CP WAX 52 CB, Varian) was used, consisting of polyethylene (100 %), with dimensions of 30 m ×0.32 mm ×0.25 µm. The temperature of the detector was 250 °C and injector was 200 °C.



At PD40, the pups were anesthetized with ketamine (90 mg/kg) and xylazine (9 mg/kg). After corneal reflex absence, surgery for salivary glands collection was performed. Left side salivary glands (Parotid gland and Submandibular gland) were assigned for some analyses (Lipid peroxidation, Nox metabolites evaluation by Griess reagent assay, and *Antioxidant Capacity against Peroxils Radicals* – ACAP). Right side salivary glands (PG and SMG) were perfused (heparinezed saline and buffered paraformaldehyde 4%) and post-fixed with a solution of

2.3. Collection of salivary glands

Fig. 1. Methodological summary of intoxication period, collection of major salivary glands and analyses. Pregnant *wistar* rats were divided into Control Group (n = 4) and EtOH group (n = 4). Both groups received, in addition to water and standard feeding, intragastric gavage on the 6th, 7th and 8th days of gestation (GD6, GD7 and GD8). (1). After 40 days of offspring, the following salivary glands (Parotid gland[PG] and Submandibular gland [SMG]) and stimulated saliva were collected from these animals for determination of total protein concentration and amylase activity (2); Analyses of oxidative biochemistry based on Oxidative Stress (3), through NOx metabolites evaluation, Malondialdehyde (MDA) and Antioxidant Capacity Against Peroxyl Radicals (ACAP); cytokeratin-19 (CK-19), and Vimentin (Vim) were analyzed by immunohistochemical analyses (4), Morphometric analyses of acini, ducts, parenchyma and stroma total area were also performed (5).

formaldehyde 6%.

2.4. Collection of saliva and Salivary analyses

After anesthetic procedures, an intraperitoneal injection of 1 mg/kg of pilocarpine dissolved in distilled water was performed for salivation stimulation (4 pups per group). The objective of pilocarpine was to stimulate salivary secretion in both groups at same condition. Pilocarpine is commonly used for cases of xerostomia due to its effects on muscarinic M3 receptors and it was used due to spontaneous saliva collection limitations regarding amount of collected saliva [20]. The saliva produced dripped in plastic tubules maintained in dry ice during saliva collection period (10 min). The samples were centrifuged at 4000 rpm at 4 °C for 5 min. Supernatants were stored at − 80 °C for amylase and total protein content analyses. The animals used for saliva stimulation were not assigned for oxidative stress and immunohistochemistry assays.

Amylase test-kit was used to determine alpha-amylase concentration (BIOCLIN™, QUIBASA, Química basica Ltda., Belo Horizonte, Minas ´ Gerais, Brazil) previously a protein concentration adjustment of collected samples. Amylase activity (µg of starch/µg of salivary protein) was determined by the method described by Caraway et al. 1959 [21]. using a solution of starch as standard. The color intensity was measured at 660 nm on a Beckman DU-800 spectrophotometer (Beckman, Fullerton, CA).

2.5. Immunohistochemistry assay

Samples were post-fixed in formaldehyde 6% for 24 h and then, they were dehydrated in increasing ethanol concentrations, cleared in xylene, and embedded in Paraplast. Five µm sections were deparaffinized and rehydrated in battery with decreasing concentration of alcohol for immunohistochemistry analysis. After rehydration, sections were rinsed in PBS (0.1 M) for 5 min. Antigen recovery was performed with a 60 °C citrate buffer solution (pH 6.0), previously heated for 20 min. Endogenous peroxidase activity inhibition using hydrogen peroxide solution (1%, H_2O_2) with methanol solution was done for 20 min. Following endogenous peroxidase inhibition, sections were rinsed with a PBS/ Tween solution (0.1 M) for five minutes and incubated in horse serum (10 %) with bovine albumin serum (3%, BSA) for 2 h. Sections without another rinsing were incubated in primary antibodies Cytokeratin-19 (CK-19) (1:50, Dako), and antivimentin (VIM) (1:50, Bioss) overnight. Subsequently, the sections were rinsed with PBS/Tween for 5 min (three times) and incubated with secondary antibody horse anti-mouse (Vector Laboratories) for 2 h. Diaminobenzidine (Sigma Chemical Corp., St. Louis, USA) was used as the chromagen after rinsing sections with PBS (0.1 M) and the sections were counterstained with Mayer's hematoxylin (Sigma Chemical Corp., St. Louis, USA)

Immunohistochemical analysis of two slides per animal was performed, one submandibular and one parotid gland. The antibody immunostaining was measured throughout the glandular parenchyma, by evaluation of the extent of the area (µm²). Photomicrographs of five randomly selected areas for each sample were acquired with Leica DM500 microscope with IC50 HD® camera attached to it (Leica microsystems, Heerburg, Switzerland), with a magnification of 40 × . Areas stained by DAB were separated and segmented using "color deconvolution plug-in" (Gabriel Landini, http://www.dentistry.bham.ac.uk/landinig/software/software.html) from ImageJ software version 1.33–1.34 (NIMH, NIH, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/) [22]. After image segmentation, the total immunostained area was measured according to the protocol proposed by Fagundes et al. 2016 [10].

2.6. Morphometric analyses

To link the results of immunohistochemistry assay, morphometric evaluation was performed. Five micrometers sections, colored in hematoxilin and eosin, were obtained for morphometric analyses considering area of parenchyma, stroma, acini, and ducts following the protocol of Merlo et al. 2010 [23]. The analyses of two slides per animal were performed using Color Threshold function of Fiji ImageJ software version 2.4 selecting the ROI (region of interest) for each parameter. After setting scale of photomicrographs, brightness adjustments in color threshold function delimited salivary glands parenchyma (acini and ducts) and stroma total area. For ducts measurement, freehand selection tool was used for measurement of each duct present in image. A sum of each duct area was obtained and subtracted from previously total parenchyma area, resulting in total area of acini presented in the same image. All measurements were described in µm².

2.7. Oxidative stress and Nitric oxide (NOx) metabolites by Griess reagent assay

Parotid and submandibular glands were rinsed in saline solution and stored at − 80 °C. Samples were submitted to sonic disintegration in 20 mM Tris-HCl buffer, pH 7.4, at 4 °C (approximate concentration of 1 g/ mL). For analysis of antioxidant pathway, Antioxidant Capacity Against Peroxils radicals (ACAP) was chosen.

For ACAP analysis [24], reaction buffer (127.5 µL) containing 30 mM HEPES (pH 7.2), 200 mM KCl and 1 mM MgCl2 was added to the wells with samples. In three of the six wells of each sample, 7.5 µL of 2, 2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich) was added. Ultrapure water was pipeted in other 3 wells as blank. Fluorescence microplate reader (Victor 2, Perkin Elmer) made the first analysis at 35 °C. Immediately before the microplate reading, 10 µL of the fluorescent probe 2′,7′ dichlorofluorescein diacetate (H2DCF-DA) was added into microplate wells with a final concentration of 40 µM. H2DCF-DA is cleaved by esterases that are present in sample supernatants. Thereafter, the nonfluorescent compound H2DCF is oxidized by ROS to the fluorescent compound DCF, which is detected in wavelength of 525 nm. The temperature decomposition of ABAP and ROS formation was monitored for 60 min, with readings every 10 min.

Nitric oxide metabolites (a by-product of the oxidation of nitric oxide) were evaluated according to the method of Griess et al. [25]. Briefly, aliquots of the homogenates were incubated at room temperature for 10 min with equal volumes of Griess solution (1% sulfanilamide in 1% H_3PO_4:0.1 % N-(1-

naphthyl)-thylenediamine dihydrochloride: distilled water, 1:1:1) at room temperature for 10 min. The absorbance was recorded at 560 nm and compared to those of standard solutions of sodium nitrite. Results were normalized according to protein content of the samples [18] and, then, were expressed as percentage of the control group.

The levels of lipid peroxidation of the samples were determined according to Esterbauer and Cheeseman (1990) [26], quantifying the chromogenic compound produced by the interaction between malondialdehyde (MDA) and N-methyl-2-phenylindole (NMFI) in the presence of methanesulfonic acid. The samples were centrifuged at 5600 rpm for 10 min. A solution of 325 µL of NMFI (10.3 mM in acetronitrile) diluted in methanol (650 µL) and 75 µL of methanesulfonic acid were added to 100 µL of samples homogenates or standard solutions with MDA and incubated for 40 min at 45 ◦C. Absorbance was recorded at 570 nm. Results of levels were normalized according to protein content of the samples [27] and, then, were expressed as percentage of the control group.

2.8. Statistical analyses

The mean values were obtained from the control and EtOH groups, among parotid and submandibular glands for each analysis, biochemical or morphometric analysis. The Shapiro-Wilk test was used for normality distribution of data. The Student *t*-test was applied for parametric data and the Mann-Whitney *U* test for nonparametric data. Analysis of variance test for offspring weight analysis was performed. All analyses were tabulated in and statistical significance *p* values was under 0.05.

3. Results

3.1. BAC, experimental animals and body weight

The BAC measured one hour after the three-day EtOH binge-drinking protocol (GD8) reached 177 ± 18.37 mg/dL. Two pregnant rats were used for BAC measurement and their respective offsprings were not taken into account for further analyses. The number of animals per litter was 8.33 ± 1.26. The animal's body weight measured from one day after birth until PD40 (first to the sixth week) were not significantly different between groups (Fig. 2). There were no deaths during the EtOH binge- drinking protocol.

3.2. Oxidative stress and Griess reagent assays, salivary total protein concentration and amylase activity

In the PG, NOx metabolites levels were not different between groups (Control: 100 ± 12.4% / EtOH: 119.7 ± 26.75%; p = .5806) while MDA levels were significantly higher in the EtOH group (Control: 100 ± 9.103% / EtOH: 199.4 ± 33.49%; p = .0212). The ACAP analysis also revealed a significant difference between groups (Control: 100 ± 17.37% / EtOH: 18.46 ± 9.522%; p = .0068).

Regarding the SMG, both NOx metabolite levels (Control: 100 ± 10.42% / EtOH: 188.8 ± 32.45%; p = .0178) and MDA were found significantly higher in the EtOH group (Control: 100 ± 13.39% / EtOH: 160.2 ± 10.14%; p = .0231); however, no difference between groups was found for the ACAP (Control: 100 ± 18.33% / EtOH 33.22 ± 20.63%; p = .0590) (Fig. 3).

The salivary total protein concentration was significantly lower in the EtOH group (Control: 625.2 ± 55.09µg/mL / EtOH: 393.5 ± 56.53µg/mL; p = .0261) and the amylase activity was also found significantly lower in the control group (Control: 0.1338 ± 0.01034 µg/ µg of protein / EtOH: 0.1964 ± 0.04114 µg/µg of protein; p = .0483) (n = 4) (Fig. 4).

3.3. Morphometric analyses In the PG, significant differences were found in all measurements (n = 8). A decrease of total parenchymal area (Control: 42.55 ± 0.6423µm^2 / EtOH: 37.29 ± 1.511µm^2; p = .0064) and an increase of total stromal area were found in the EtOH group (Control: 4.359 ± 0.7253µm^2 / EtOH: 8.147 ± 0.9164µm^2; p = .0059) (Fig. 5). A reduction of total acinar area (Control: 40.19 ± 0.5448µm^2/ EtOH: 36.4 ± 1.56µm^2; p = .0379) and ducts (Control: 2.354 ± 0.3248µm^2 / EtOH: 0.8816 ± 0.07245µm^2; p = .0006) were observed in the EtOH group.

Regarding the SMG. EtOH group showed a significant increase of total stromal area (Control: 7.442 ± 0.5024µm^2 / EtOH: 11 ± 0.490µm^2; p = .002); however, no significant differences between groups were observed for acini (Control: 38.6 ± 0.9798µm^2 / EtOH: 35.96 ± 0.8976µm^2; p = .0672), ducts (Control: 34.49 ± 1.023µm^2 / EtOH: 31.46 ± 1.091µm^2; p = .0624), and parenchyma (Control: 4.108 ± 0.5483µm^2 / EtOH: 4.499 ± 0.4297µm^2; p = .5832) (Fig. 6).

3.4. Immunohistochemistry analyses

The immunostaining of CK-19 and Vimentin were not different between groups. Figs. 7 and 8 respectively show CK-19 staining and Vimentin analyses in Control and EtOH groups for both PG and SMG. Samples were collected from 8 animals of each group.

4. Discussion

This study investigated the potential biochemical, morphologic, and functional effects of EtOH binge drinking during pregnancy on offspring rats' salivary glands. The results showed that three-day EtOH consumption followed by four-day abstinence is associated with morphologic alterations in salivary glands, particularly in PG, which seem related to oxidative stress.

According to a WHO report, the current absolute number of drinkers has increased, particularly among 15 to 19-years-old women [1]. The National Institute of Alcohol Abuse and Alcoholism also reported that 20%–30% of women eventually drink during pregnancy [28]. Therefore, prenatal EtOH exposure is a risk for developing disorders in the central nervous system (CNS), which is strictly related to the major salivary glands [28–30].

Some authors investigated the prenatal effects caused by the consumption of 3 g/kg of EtOH during pregnancy and demonstrated that a BAC of 150−200 mg/dl promoted motor deficit in offspring rats [18,31]. Our research group has shown that the consumption of 3 g/kg/day of EtOH by adult female rats may affect PG and SMG morphology and oxidative balance [10]. In this study, the BAC levels reached 177 mg/dl after using a three-day EtOH binge-drinking protocol similar to other published research [18]; thus, this protocol seems adequate for further salivary glands evaluation.

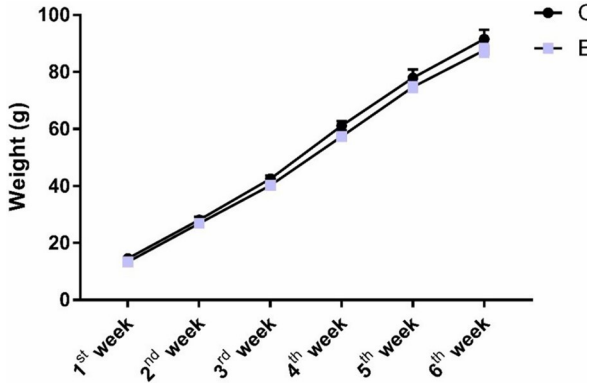


Fig. 2. Body weight of Control and EtOH offspring groups. Weekly body weight measurements were performed after postnatal day 7, from 1st to 6th week. ANOVA one way with repeated measures and Tukey's post hoc test, $p < 0.05$.

cells [30,31]. CK-19 and Vimentin are related to the ductal system's cytoskeleton and myoepithelial cells,

Considering that EtOH consumption may affect the structure and physiology of salivary glands, this study aimed to investigate the effect of 3 g/kg/day of EtOH following a three-day binge-drinking protocol on PG and SMG. Chronic EtOH consumption has been associated with the unbalance of CK-19 and α-SMA proteins, fundamental for acini and duct establishment and salivary secretion within parenchyma [11]. CK-19 expression is related to granular ducts, striated ducts, and the luminal cells in excretory ducts. Vimentin is an essential protein associated with myoepithelial cells and expressed by mesenchymal cells and some ductal

respectively [32]. Corroborating these findings, it has been demonstrated that prolonged EtOH consumption caused high apoptosis activity and differences in the parenchymal and stromal volumes of salivary glands [10].

SMG and PG are major salivary glands and play an essential role in producing saliva components [6,8,23]. Due to differences in morphology (mucous and serous acini) and salivary secretion, PG is more vulnerable to toxic agents and showed increased lipid peroxidation and cell death when exposed to the remarkably toxic methylmercury [33,34]. Therefore, PG may be distinctively affected by toxic agents due to its increased protein synthesis functions [30,35].

Another study reported that the exposure to EtOH binge drinking resulted in Caspase-3 overexpression and lipid peroxidation in PG, which indicates that these glands may be damaged by oxidative stress, albeit disorders associated with cell death mechanism control are evident This study demonstrated that oxidative stress affects PG and

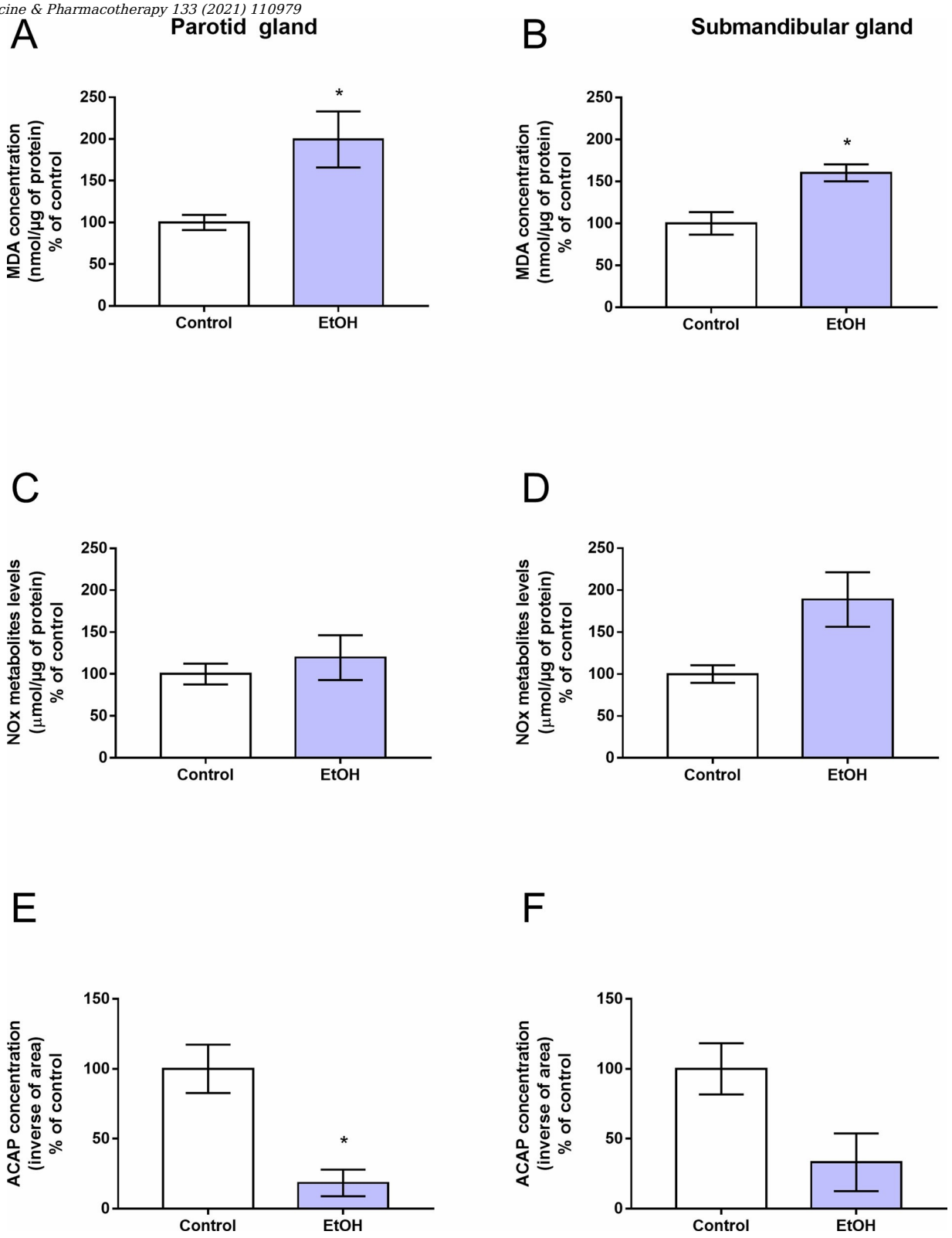


Fig. 3. Biochemical analyses of Parotid and Submandibular Glands. Letters A to F show graphs representing oxidative stress assays of PG and SMG samples. (A) MDA concentration assay in parotid gland (B) MDA concentration in submandibular gland (C) NOx metabolite levels in parotid glands (D) NOx metabolite levels in submandibular gland (E) ACAP in parotid glands (F) ACAP in submandibular glands. Analyses were performed with 8 animals for each group (control/EtOH).
Differences are represented by values of Mean ± SEM in % of control. Student's t test. *Statistical significance ($p < 0.05$).

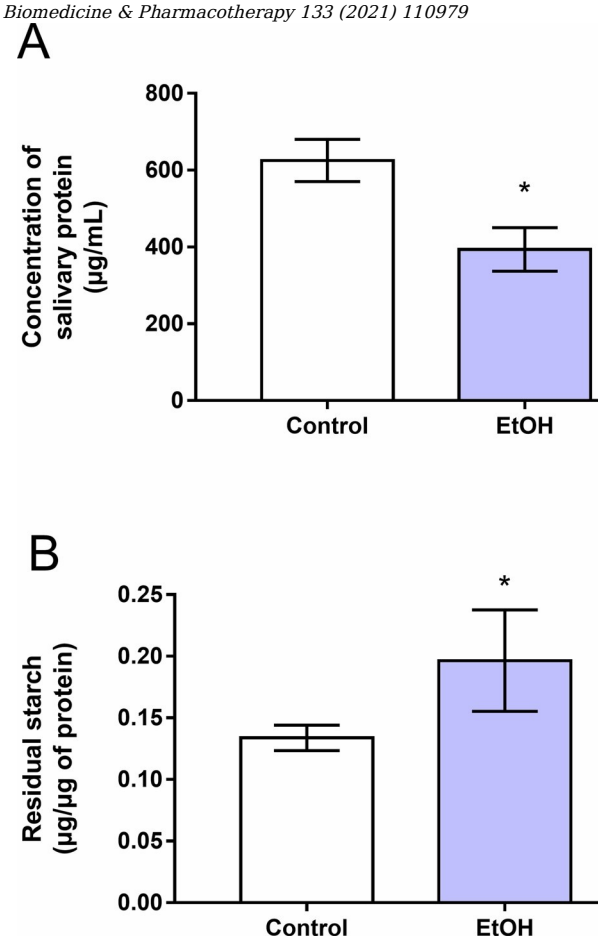


Fig. 4. Salivary protein concentration andamylase activity**.** Letter A and B represent total protein concentration and amylase activity of the experimental groups, respectively. Student *t*-test $p < 0.05$.

SMG and may lead to morphologic alterations. In contrast, some authors reported functional changes related to ethanol consumption [36], such as reduced salivary flow, hyperfunction of ductal cells due to sodium and potassium unbalances, and hypertrophic alterations of both acinar and ductal cells [37,38]. These changes are diagnosed as sialosis (or sialadenosis) and are related to the accumulation of granules of different shapes and sizes occupying the cytoplasm's full extent. Both acinar and ductal cells are associated with lipids accumulation and are probably related to fatty acids metabolism disorders and autonomic innervation of glands [38]. However, the absence of a significant difference between groups on immunohistochemistry data might be related to the long period without EtOH consumption and potential regulatory mechanisms to repair the damage [39].

EtOH induces detrimental pleiotropic effects on several body tissues and glands through excitotoxicity, neuroinflammation, and oxidative stress [40]. The EtOH metabolism generates the overproduction of reactive species through the up-regulation of multiple pro-oxidant pathways and direct production by alcohol dehydrogenase (ADH) 3 and 4, catalase, and CYP2E1 [41]. Also, the overexpression of inducible nitric oxide synthase (iNOS) generates more reactive nitrogen species (RNS). In contrast, EtOH also impairs the mitochondrial activity that reduces antioxidant defenses (i.e., glutathione) [42,43].

The increased stroma and the decrease of parenchyma, acini, and ducts in PG, could be considered an attempt to repair damages not related to oxidative stress [11]. In this study, EtOH was administered at GD6, GD7, and GD8. These periods correspond to gastrulation and neurulation phases [18], in which the innervations of several tissues are developed, including the salivary glands (submandibular ganglion, facial nerve, and hypoglossal nerve) [30,39]. Therefore, specific damage to parasympathetic innervation may lead to recurrent or permanent PG deficits.

Some authors have demonstrated that acetylcholine, which is the primary neurotransmitter released from parasympathetic nerves, is responsible for several phases of salivary glands tubulogenesis and branching morphogenesis [39,44,45]. Its interaction with muscarinic M1 receptors is essential to stimulate growth factors for epithelial differentiation and growth of glands. The epidermal (EGF), fibroblast

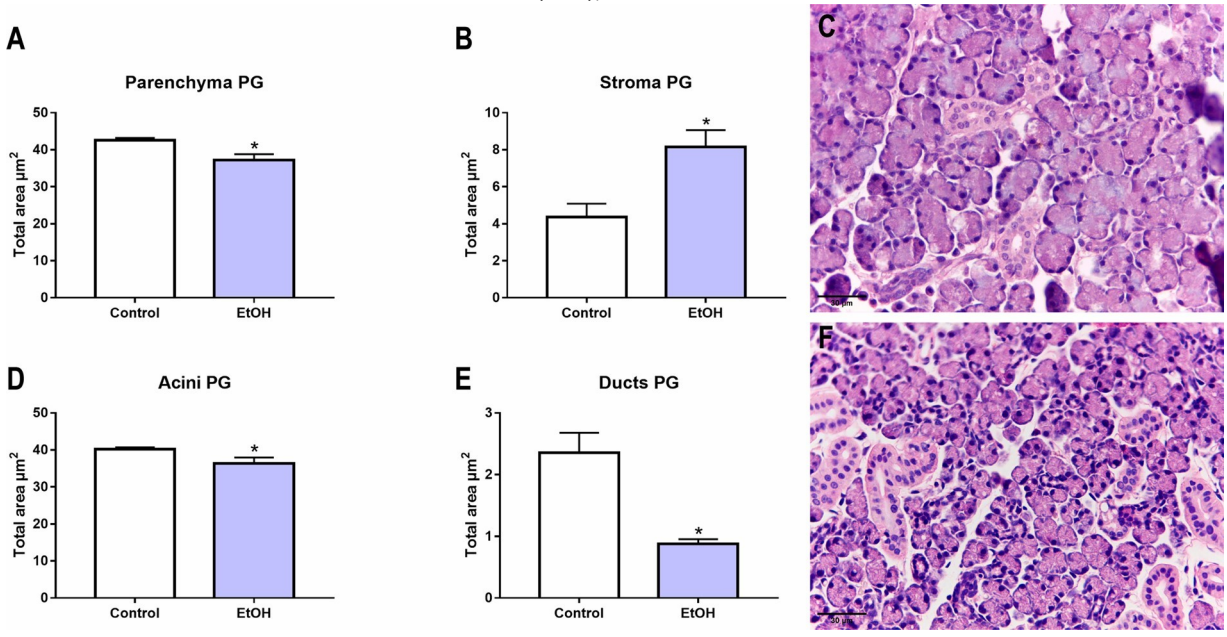


Fig. 5. Morphometric analyses of PG. Figure represents graphs of statistical analyses among parenchyma, acini, stroma, and ducts variables. (A) Total area (μm²) of parotid parenchyma (B) Total area (μm²) of parotid stroma (C) Representative photomicrograph of parotid control group. Scale bar: 30 μm. (D) Total area (μm²) of parotid acini (E) Total area (μm²) of parotid ducts. Representative photomicrograph of parotid EtOH group. Scale bar: 30 μm. Student's t test. *Statistical significance ($p < 0.05$).

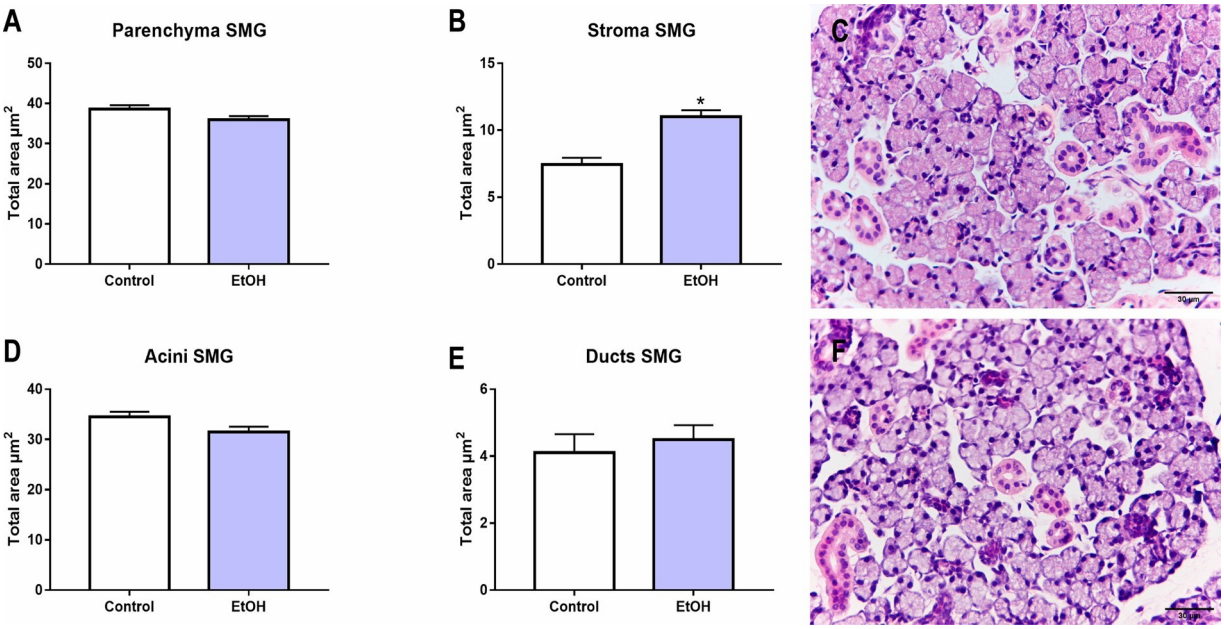


Fig. 6. Morphometric analyses of Submandibular Gland. Figure represents graphs of statistical analysis among parenchyma, acini, stroma, and ducts variables. (A) Total area (μm²) of submandibular parenchyma (B) Total area (μm²) of submandibular stroma (C) Representative photomicrograph of submandibular control group. Scale bar: 30 μm. (D) Total area (μm²) of submandibular acini (E) Total area (μm²) of submandibular ducts. Representative photomicrograph of submandibular EtOH group. Scale bar: 30 μm. Differences are represented by values of Mean ± SEM. Student's t test. *Statistical significance ($p < 0.05$).

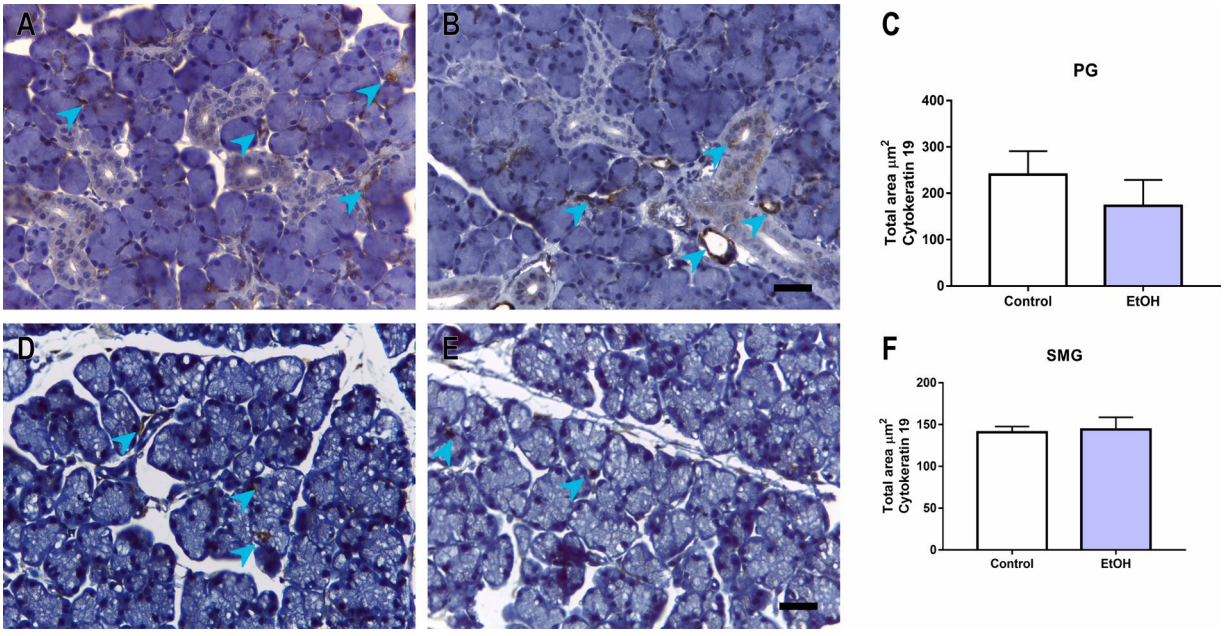


Fig. 7. Photomicrographs representing CK-19 immunohistochemistry assay of Parotid and Submandibular glands. (A) Representative photomicrograph of CK-19 immunohistochemistry for parotid control group. Blue arrowheads: area of diaminobenzidine (DAB) staining. (B) Representative photomicrograph of CK-19 immunohistochemistry for parotid EtOH group. Blue arrowheads: area of diaminobenzidine (DAB) staining. (C) Total area of DAB staining for parotid glands (μm²). (D) Representative photomicrograph of CK-19 immunohistochemistry for submandibular control group. (E) Representative photomicrograph of CK-19 immunohistochemistry for submandibular EtOH group. (E) Total area of DAB staining for submandibular glands(μm²). Analyses were performed with 8 animals for each group (control/EtOH). Differences are represented by values of Mean ± SEM. Student's t test. *Statistical significance ($p < 0.05$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(FGF), and platelet-derived growth factors (PDGF) are secreted by the acetylcholine-M1 signaling pathway and are essential to salivary glands proliferation [46,47]. Thus, further studies evaluating EtOH toxicity and potential impairments on the acetylcholine-signaling pathway are suggested.

One of the main metabolic products of EtOH consumption is known as acetaldehyde and figures as another relevant aspect for further evaluation [41]. This substance is freely present in the placenta after EtOH binge drinking and can decrease both blood flow and placenta growth [29]; thus, potential impairment of cell migration and amino acid transportation may jeopardize neurological development and lead to motor or intellectual deficits. Therefore, damage in the autonomic

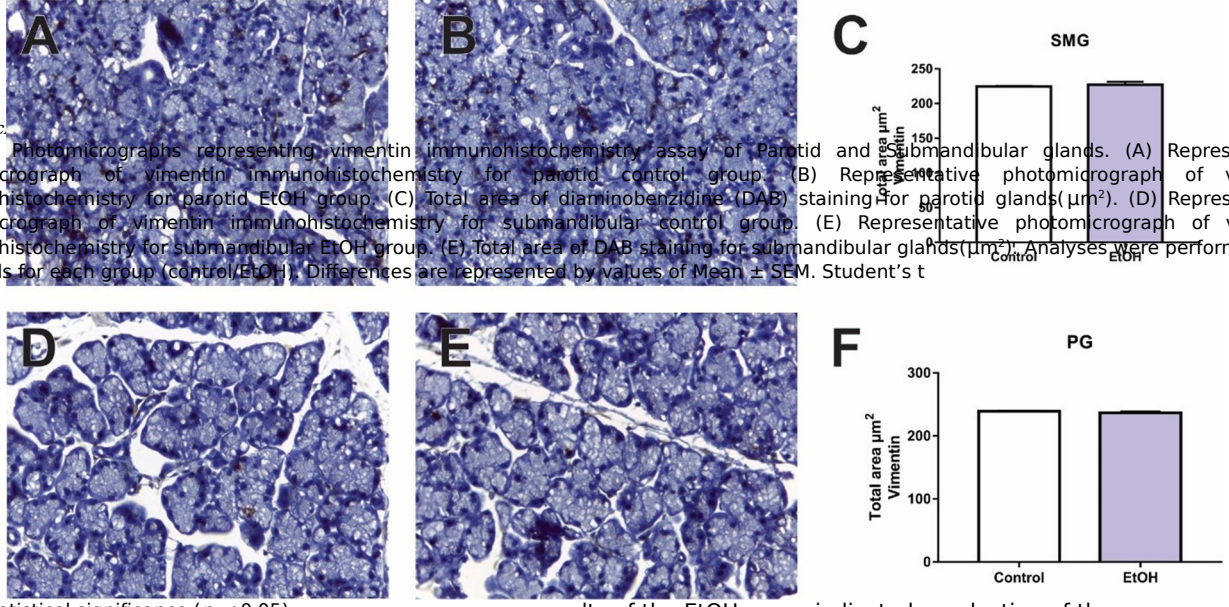


Fig. 8. Photomicrographs representing vimentin immunohistochemistry assay of Parotid and Submandibular glands. (A) Representative photomicrograph of vimentin immunohistochemistry for parotid control group. (B) Representative photomicrograph of vimentin immunohistochemistry for parotid EtOH group. (C) Total area of diaminobenzidine (DAB) staining for parotid glands(μm^2). (D) Representative photomicrograph of vimentin immunohistochemistry for submandibular control group. (E) Representative photomicrograph of vimentin immunohistochemistry for submandibular EtOH group. (F) Total area of DAB staining for submandibular glands(μm^2). Analyses were performed with 8 animals for each group (control/EtOH). Differences are represented by values of Mean ± SEM. Student's t test. *Statistical significance ($p < 0.05$).

innervation development and progenitor cell differentiation could affect morphogenesis and tubulogenesis of salivary glands [45,46].

Salivary protein content includes enzymes, glycoproteins, mucins, polypeptides, statins, proline-rich peptides, and several other proteins responsible for the innate immune response to digestion functions [48]. Cystatins are a group of multigene proteins that includes the α-amylase and is crucial to innate immune response and carbohydrate hydrolysis. The total salivary protein content contains approximately 50 % of amylase [49]. In this study, EtOH consumption decreased the salivary total protein concentration and increased amylase activity. EtOH was reported to reduce amylase activity [50]. and even evaluating its indirect effects on salivary glands, amylase activity remained reduced after prenatal EtOH exposure. Although the amylase's physiologic production in offspring rats remains present, the lack of amylase and other proteins needs to be addressed.

Regarding the oxidative parameters investigated in this study, lipid peroxidation, amylase activity alteration, and decreased antioxidant capacity was found in the salivary glands. The concentration of EtOH metabolite presumably caused indirect damages in the salivary glands [51]. EtOH was suggested to increase lipid peroxidation in salivary glands, mostly PG [52]. In comparison to SMG, PG presents a higher aerobic metabolism capacity that indicates differences in the regulatory mechanisms [53]. Moreover, EtOH consumption reduces antioxidant enzymes (such as SOD and GPx) in the PG tissues and damages the ROS membrane [52].

The ACAP analysis allowed the evaluation of the antioxidant response of salivary gland samples by inducing peroxyl radicals [24]. This practical and efficient method has been previously used to investigate biochemical changes in salivary glands exposed to toxic metals [33]; thus, ACAP was used in this study to generally assess the cellular response "without" (without ABAP) and "with ROS induction" (with ABAP) [24]. Through ABAP addition, the ROS induction allows the analysis of these radicals' interception by the cellular antioxidant system [24]. Another point is that peroxyl radicals are strongly related to malonaldehyde production since alkyl radicals interact with peroxyl generating MDA [54]. In this sense, MDA is associated with DNA damage through guanine-link interactions that lead to cell death [54]. In addition to lipid peroxidation, the results of the EtOH group indicated a reduction of the peroxyl radicals' interception response in the PG that constitutes the oxidative stress event and seems related to the morphometric alterations observed in the parenchyma of the salivary glands.

It has been argued that the DCFH probe used in this study has inappropriate specificity for identifying antioxidant enzymes, albeit it has been reported as antioxidant reactions indicator [55]. DCFH does not directly react with H_2O_2, but directly and indirectly reacts with others oxidant agents such as hydroxyl radicals (·OH), oxygen (O_2), cytochrome C, and iron (Fe^{2+}) [28]. Even though the results showed a decreased antioxidant capacity in salivary glands, further evaluations are needed to address well-known antioxidant enzymes in the tissue homogenate such as catalase, GPx, and SOD [55–58].

Also, oxidative stress is a complex interaction between oxidant challenge derived from oxidant stress and external oxidant molecules, nutritional antioxidant system, and endogenous redox regulation that may lead to imbalances [59]. As an example of an endogenous oxidant reaction involved in physiological functions, nitric oxide acts in the vasodilation of mature salivary glands through parasympathetic stimulation and is also involved in salivary protein secretion sympathetic and parasympathetic stimulation [30]. The autonomic nervous system's stimuli are associated with the neuronal vasoactive intestinal peptide (VIP), which is an essential neuropeptide for salivary glands secretion and has been significantly related to tubulogenesis [44–46]. During salivary glands development, VIP mediates tubular cells' proliferation during branching morphogenesis through the AMPc/protein kinase A signaling pathway. Both extracellular Ca^{2+} and endoplasmic reticulum-derived influxes increase nitric oxide synthase in ductal cells and generate tubular enlargement [45].

As stated before, the nitrite oxide-signaling pathway is relevant to the salivary glands' development and function [45]. Several variables may influence salivary glands development by enhancing or decreasing the oxidative stress or teratogenic factors related to EtOH consumption. Therefore, research on the role of oxidative stress on detrimental effects in salivary glands' morphology and whether it is modulated by other molecules such as acetaldehyde or microvascular deficits are encouraged [13].

Morphological alterations were mainly observed in PG even 50 days after EtOH consumption. In this

context, pregnancy may lead to changes in the salivary glands of offspring rats and functional disorders [60]. Further research with more extended prenatal EtOH exposure may lead to different oxidative or physiologic disorders in major salivary glands.

5. Conclusions

We showed that one EtOH binge drinking episode during a critical stage of gestational period is linked to morphometric alterations, oxidative stress, and functional changes in salivary glands of offspring rats.

Authors contributions

ROF, LPMF and RRL: conceptualization, study design, analysis of results, manuscript elaboration; WABA, LOB, KMB: methodology, analysis of results, manuscript elaboration. SMA, JJVP, MECL, CSFM: analysis of results and manuscript elaboration.

Ethical approval

All procedures were conducted under compliance with policies of Federal University of Para Ethics Committee of Animals Use (CEUA N´ º 6478190717) and following the guidelines of NIH *Guide for the Care and Use of Laboratory Animals*

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Declaration of Competing Interest

The authors report no declarations of interest.

References

[1] W.H. Organization, Global Status Report on Alcohol and Health 2018, 2018.
[2] W.H. Organization, W.H.O.M.o.S.A. Unit, Global Status Report on Alcohol and Health, World Health Organization, 2014.
[3] A. Ornoy, Z. Ergaz, Alcohol abuse in pregnant women: effects on the fetus and newborn, mode of action and maternal treatment, Int. J. Environ. Res. Public Health 7 (2) (2010) 364–379.
[4] S. Alcohol research: current reviews editorial, drinking patterns and their definitions, Alcohol Res. 39 (1) (2018) 17–18.
[5] M. Stahre, J. Roeber, D. Kanny, R.D. Brewer, X. Zhang, Contribution of excessive alcohol consumption to deaths and years of potential life lost in the United States, Prev. Chronic Dis. 11 (2014) E109.
[6] O. Amano, K. Mizobe, Y. Bando, K. Sakiyama, Anatomy and histology of rodent and human major salivary glands: -overview of the Japan salivary gland society- sponsored workshop, Acta Histochem. Cytochem. 45 (5) (2012) 241–250.
[7] R. Taga, A. Sesso, Ultrastructural studies on developing parotid gland of the rat at early postnatal periods, Archivum histologicum Japonicum = Nihon soshikigaku kiroku 42 (4) (1979) 427–444.
[8] N. Waszkiewicz, B. Zalewska-Szajda, A. Zalewska, M. Waszkiewicz, S.D. Szajda, B. Repka, A. Szulc, A. Kepka, A. Minarowska, S. Chojnowska, B. Konarzewska, J. R. Ladny, U. Kowzan, K. Zwierz, Decrease in salivary lactoferrin output in chronically intoxicated alcohol-dependent patients, Folia Histochem. Cytobiol. 50 (2) (2012) 248–254.
[9] S. Murakami, B.L. Mealey, A. Mariotti, I.L.C. Chapple, Dental plaque–induced gingival conditions, J. Clin. Periodontol. 45 (S20) (2018) S17–S27.
[10] N.C. Fagundes, L.M. Fernandes, R.S. Paraense, P.M. de Farias-Junior, F.B. Teixeira, S.M. Alves-Junior, J. Pinheiro Jde, M.E. Crespo-Lopez, C.S. Maia, R.R. Lima, Binge drinking of ethanol during adolescence induces oxidative damage and morphological changes in salivary glands of female rats, Oxid. Med. Cell. Longev. 2016 (2016) 7323627.
[11] L.M. Fernandes, F.B. Teixeira, S.M. Alves-Junior, J. Pinheiro Jde, C.S. Maia, R. R. Lima, Immunohistochemical changes and atrophy after chronic ethanol intoxication in rat salivary glands, Histol. Histopathol. 30 (9) (2015) 1069–1078. [12] K.J. Dal Pra, A. de Fatima Moraes da Silva, A.H. Chaves-Neto, A.M.P. Soubhia,´ Effects of cachaça, a typical Brazilian alcoholic beverage, on submandibular glands of rats: a histomorphometric and biochemical study, Clin. Oral Investig. (2020). [13] K.V. Shevchenko, G.A. Yeroshenko, S. Yakushko O, K.S. Kazakova, D. R. Kramarenko, Morphometric description of the exchange segment of microvasculature of rats' salivary glands in normal conditions and chronic ethanol intoxication, Wiad. Lek. 72 (3) (2019) 323–326.
[14] K.V. Shevchenko, G.A. Yeroshenko, V. Vilkhova O, D.R. Kramarenko, S. Yakushko O, A.I. Yachmin, Remodeling of the duct system of the rat submandibular salivary glands in chronic ethanol intoxication, Wiadomosci lekarskie (Warsaw, Poland : 1960) 73 (1) (2020) 128–133.
[15] T.S. Naimi, L.E. Lipscomb, R.D. Brewer, B.C. Gilbert, Binge drinking in the preconception period and the risk of unintended pregnancy: implications for women and their children, Pediatrics 111 (5 Pt 2) (2003) 1136–1141.
[16] C.M. O'Leary, Alcohol and pregnancy: do abstinence policies have unintended consequences?, Alcohol and alcoholism (Oxford, Oxfordshire 47 (6) (2012) 638–639.
[17] N.R. Council, Guide for the Care and Use of Laboratory Animals, National Academies Press, 2010.
[18] U.B. Schambra, J. Goldsmith, K. Nunley, Y. Liu, S. Harirforoosh, H.M. Schambra, Low and moderate prenatal ethanol exposures of mice during gastrulation or neurulation delays neurobehavioral development, Neurotoxicol. Teratol. 51 (2015) 1–11.
[19] L. Quiros-Ter´ ron, L.A. Ar´ raez-Aybar, J. Murillo-Gon´ zalez, C. De-la-Cuadra-Blanco,´ M.C. Martínez-Alvarez, J.V. Sanz-Casado, J.R.´ Merida-Velasco, Initial stages of´ development of the submandibular gland (human embryos at 5.5–8 weeks of development), J. Anat. (2019).
[20] T. Narita, B. Qi, M. Murakami, H. Sugiya, Pilocarpine induces the residual secretion of salivary fluid in perfused submandibular glands of rats, PLoS One 14 (8) (2019) e0221832.
[21] W.T. Caraway, A stable starch substrate for the determination of amylase in serum and other body fluids, Am. J. Clin. Pathol. 32 (1959) 97–99.
[22] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, Fiji: an open-source platform for biological-image analysis, Nat. Methods 9 (7) (2012) 676–682.
[23] C. Merlo, L. Bohl, C. Carda, M.E. Gomez de Ferraris, M. Carranza, Parotid sialosis: morphometrical analysis of the glandular parenchyme and stroma among diabetic and alcoholic patients, J. Oral Pathol. Med. 39 (1) (2010) 10–15.
[24] L.L. Amado, M.L. Garcia, P.B. Ramos, R.F. Freitas, B. Zafalon, J.L.R. Ferreira, J. S. Yunes, J.M. Monserrat, A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: application to evaluate microcystins toxicity, Sci. Total Environ. 407 (6) (2009) 2115–2123.
[25] L. Green, P.Goldman Tannenbaum, Nitrate Synthesis in the Germfree and Conventional Rat, 212, 1981, pp. 56–58, 4490.
[26] H. Esterbauer, K.H. Cheeseman, 42] Determination of Aldehydic Lipid Peroxidation Products: Malonaldehyde and

4-Hydroxynonenal, Methods in Enzymology, Elsevier, 1990, pp. 407–421.
[27] M.M.J.Ab. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1–2) (1976) 248–254.
[28] S.E. Maier, J.R.J.A.R. West, Health, drinking patterns and alcohol-related birth defects, Alcohol Res. Health 25 (3) (2001) 168–174.
[29] A.N. Bukiya, A.M. Dopico, Fetal cerebral circulation as target of maternal alcohol consumption, alcoholism, Clin. Exp. Res. 42 (6) (2018) 1006–1018.
[30] G.B. Proctor, G.H. Carpenter, Regulation of salivary gland function by autonomic nerves, Autonomic Neurosci.: Basic Clin. 133 (1) (2007) 3–18.
[31] J.M. Gohlke, W.C. Griffith, E.M. Faustman, Computational models of ethanol- induced neurodevelopmental toxicity across species: implications for risk assessment, Birth Defects Res. B Dev. Reprod. Toxicol. 83 (1) (2008) 1–11.
[32] Y. Ogawa, Immunocytochemistry of myoepithelial cells in the salivary glands, Prog. Histochem. Cytochem. 38 (4) (2003) 343–426.
[33] L.O. Bittencourt, B. Puty, S. Charone, W.A.B. Aragao, P.M. Farias-Junior, M.C.˜ F. Silva, M.E. Crespo-Lopez, Ad.L. Leite, M.A.R. Buzalaf, R.R. Lima, Oxidative biochemistry disbalance and changes on proteomic profile in salivary glands of rats induced by chronic exposure to methylmercury, Oxid. Med. Cell. Longev. 2017 (2017).
[34] L.A. de Oliveira Lima, L.O. Bittencourt, B. Puty, R.M. Fernandes, P.C. Nascimento, M.C.F. Silva, S.M. Alves-Junior, Jd.J.V. Pinheiro, R.R. Lima, Methylmercury intoxication promotes metallothionein response and cell damage in salivary glands of rats, Biol. Trace Elem. Res. 185 (1) (2018) 135–142.
[35] A. Walz, K. Stuhler, A. Wattenberg, E. Hawranke, H.E. Meyer, G. Schmalz, M. Bluggel, S. Ruhl, Proteome analysis of glandular parotid and submandibular- sublingual saliva in comparison to whole human saliva by two-dimensional gel electrophoresis, Proteomics 6 (5) (2006) 1631–1639.
[36] V.R. Preedy, Alcohol and the Gastrointestinal Tract, CRC Press, 2017.
[37] L. Bohl, C. Merlo, C. Carda, M.E. Gomez de Ferraris, M. Carranza, Morphometric´ analysis of the parotid gland affected by alcoholic sialosis, J. Oral Pathol. Med. 37 (8) (2008) 499–503.
[38] G. Guan, J. Won, L. Mei, A. Polonowita, Extensive adipose replacement of the parotid glands: an unusual presentation of sialadenosis. A Case Report and Literature Review, Oral Surg. 13 (1) (2020) 41–47.
[39] V.N. Patel, M.P. Hoffman, Salivary gland development: a template for regeneration, Semin. Cell Dev. Biol. 25–26 (2014) 52–60.
[40] L.M.P. Fernandes, E.F. de Andrade, M.C. Monteiro, S.C. Cartagenes, R.R. Lima, R.´ D. Prediger, C.S.F. Maia, Chapter 20 - ethanol: neurotoxicity and brain disorders, in: R.R. Watson, S. Zibadi (Eds.), Addictive Substances and Neurological Disease, Academic Press, 2017, pp. 201–215.
[41] A.I. Cederbaum, Alcohol metabolism, Clin. Liver Dis. 16 (4) (2012) 667–685.
[42] L.M.P. Fernandes, F.R. Bezerra, M.C. Monteiro, M.L. Silva, F.R. de Oliveira, R.
R. Lima, E.A. Fontes-Júnior, C.S.F. Maia, Thiamine deficiency, oxidative metabolic pathways and ethanol-induced neurotoxicity: how poor nutrition contributes to the alcoholic syndrome, as Marchiafava-Bignami disease, Eur. J. Clin. Nutr. 71 (5)
(2017) 580–586.
[43] O. Sergent, B. Griffon, P. Cillard, J. Cillard, Alcool et stress oxydatif, Pathol. Biol.
49 (9) (2001) 689–695.
[44] S.M. Knox, I.M. Lombaert, X. Reed, L. Vitale-Cross, J.S. Gutkind, M.P. Hoffman, Parasympathetic innervation maintains epithelial progenitor cells during salivary organogenesis, Science (New York, N.Y.) 329 (5999) (2010) 1645–1647.
[45] P.I. Nedvetsky, E. Emmerson, J.K. Finley, A. Ettinger, N. Cruz-Pacheco, J. Prochazka, C.L. Haddox, E. Northrup, C. Hodges, K.E. Mostov, M.P. Hoffman, S. M. Knox, Parasympathetic innervation regulates tubulogenesis in the developing salivary gland, Dev. Cell 30 (4) (2014) 449–462.
[46] W.M. Knosp, S.M. Knox, I.M. Lombaert, C.L. Haddox, V.N. Patel, M.P. Hoffman, Submandibular parasympathetic gangliogenesis requires sprouty-dependent Wnt signals from epithelial progenitors, Dev. Cell 32 (6) (2015) 667–677.
[47] K.V. Holmberg, M.P. Hoffman, Anatomy, biogenesis and regeneration of salivary glands, Monogr. Oral Sci. 24 (2014) 1–13.
[48] L.C. Schenkels, E.C. Veerman, A.V. Nieuw Amerongen, Biochemical composition of human saliva in relation to other mucosal fluids, Crit. Rev. Oral Biol. Med. 6 (2) (1995) 161–175.
[49] A.C. Romero, C.T. Bergamaschi, D.N. de Souza, F.N. Nogueira, Salivary alterations in rats with experimental chronic kidney disease, PLoS One 11 (2) (2016) e0148742.
[50] N. Enberg, H. Alho, V. Loimaranta, M. Lenander-Lumikari, Saliva flow rate, amylase activity, and protein and electrolyte concentrations in saliva after acute alcohol consumption, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endodontol.
92 (3) (2001) 292–298.
[51] P.G. Wells, S. Bhatia, D.M. Drake, L. Miller-Pinsler, Fetal oxidative stress mechanisms of neurodevelopmental deficits and exacerbation by ethanol and methamphetamine, Birth Defects Res. C Embryo Today 108 (2) (2016) 108–130.
[52] S.C. Campos, D.A. Moreira, T.D. Nunes, P. Colepicolo, M.R. Brigagao, Oxidative˜ stress in alcohol-induced rat parotid sialadenosis, Arch. Oral Biol. 50 (7) (2005) 661–668.
[53] H. Maier, I.A. Born, S. Veith, D. Adler, H.K. Seitz, The effect of chronic ethanol consumption on salivary gland morphology and function in the rat, Alcoholism Clin. Exp. Res. 10 (4) (1986) 425–427.
[54] C. Avendano, J.C. Me˜ nendez, Chapter 4 - anticancer drugs acting via radical´ species, photosensitizers and photodynamic therapy of cancer, in: C. Avendano, J.˜ C. Men ´endez (Eds.), Medicinal Chemistry of Anticancer Drugs, Elsevier, Amsterdam, 2008, pp. 93–138.
[55] B. Kalyanaraman, V. Darley-Usmar, K.J.A. Davies, P.A. Dennery, H.J. Forman, M. B. Grisham, G.E. Mann, K. Moore, L.J. Roberts 2nd, H. Ischiropoulos, Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations, Free Radic. Biol. Med. 52 (1) (2012) 1–6.
[56] J.M. McCord, I.J.Jo.B.C. Fridovich, The reduction of cytochrome c by milk xanthine oxidase, J. Biol. Chem. 243 (21) (1968) 5753–5760.
[57] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1) (1959)
70–77.
[58] H. Aebi, 13] Catalase in Vitro, Methods in Enzymology, Elsevier, 1984, pp. 121–126.
[59] H. Sies, C. Berndt, D.P. Jones, Oxidative stress, Annu. Rev. Biochem. 86 (1) (2017)
715–748.
[60] A. Mattingly, J.K. Finley, S.M. Knox, Salivary gland development and disease, Wiley Interdiscip. Rev. Dev. Biol. 4 (6) (2015) 573–590.